National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 869a

Column Selectivity Test Mixture for Liquid Chromatography
(Polycyclic Aromatic Hydrocarbons)

This Standard Reference Material (SRM) is a mixture of three polycyclic aromatic hydrocarbons (PAHs) in acetonitrile: benzo[*a*]pyrene (BaP), 1,2:3,4:5,6:7,8-tetrabenzonaphthalene (TBN, alternate name, dibenzo[*g,p*]chrysene), and phenanthro[3,4-*c*]phenanthrene (PhPh) (see Figure 1 for structures). SRM 869a is useful for characterizing liquid chromatographic (LC) column selectivity for separation of PAHs [1,2]. Depending on the elution order of the three components, column selectivity can be predicted for complex PAH mixtures (particularly isomeric PAHs). Even though the primary use of this mixture in the past has been to characterize columns for PAH separations, applications to the assessment of column selectivity for other classes of compounds such as carotene isomers has also been demonstrated [3]. A unit of SRM 869a consists of 5 ampules, each containing 1.1 mL of the PAH mixture. The concentrations and relative responses of the components at 254 nm are listed in the Appendix, Table 1.

Expiration of Certification: SRM 869a is valid for its intended purpose until **30 September 2007**, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see *Instructions for Use*). The certification is nullified if the SRM is damaged, contaminated, or modified.

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Return of the attached registration card will facilitate notification.

NOTICE AND WARNINGS TO USERS

Toxicity: This test mixture contains small amounts of polycyclic aromatic hydrocarbons, some of which have been reported to have mutagenic and/or carcinogenic properties. Therefore, care should be exercised during handling and use (see *Instructions for Use*). Use proper methods for disposal of waste.

The coordination of the technical measurements leading to certification were performed under the direction of L.C. Sander and S.A. Wise of the NIST Analytical Chemistry Division.

Preparation and analytical determinations were carried out by L.C. Sander of the NIST Analytical Chemistry Division.

The support aspects involved in the preparation, certification, and issuance of this SRM were coordinated through the NIST Standard Reference Materials Group by B.S. MacDonald.

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Certificate Issue Date: 22 October 2002
See Certificate Revision History on Page 5

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INSTRUCTIONS FOR USE

Storage: Sealed ampoules, as received, should be stored in the dark at temperatures between 10 °C and 30 °C.

Conditions: This test mixture is intended primarily for the characterization of C_{18} columns used in the reversed-phase liquid chromatographic separation of PAHs. To compare columns on the same basis, the user should evaluate column selectivity by chromatographing the mixture isocratically under the following conditions: mobile phase, 85 % acetonitrile/water (v/v) at 25 °C ± 2 °C. Changes in both absolute retention and selectivity can occur with changes in temperature [4] and composition [5]; thus these conditions should be used for all column evaluations to enable comparisons with the results reported in this certificate.

Representative separations of the test mixture are illustrated in Figure 2 (A-E) for several different C_{18} columns. These chromatograms are examples of the five possible cases for solute elution order, and are provided as an aid for component identification. In cases where complete separation is achieved (Figure 2 (A, C, and E), BaP is the smallest peak, PhPh is intermediate in size, and TBN the largest peak when using UV detection at 254 nm (see the Appendix for peak height and peak area data).

Interpretation of Results: The elution order of the three components changes with C_{18} stationary phase type [6] as shown in Figure 3. Monomerically bonded ("monomeric") C_{18} phases (prepared using monofunctional silane reagents) have been shown to give the elution order BaP ≤ PhPh < TBN (see Figure 2 (A and B)). Stationary phases prepared using polymeric surface modification procedures ("polymeric", i.e., using trifunctional silanes in the presence of water) have the elution order PhPh < TBN ≤ BaP (Figure 2 (D and E)). Stationary phases with intermediate properties (i.e., densely loaded monomeric or lightly loaded polymeric C_{18} phases) are indicated by the elution order PhPh < BaP < TBN (see Figure 2 (C)).

A measure of stationary phase selectivity can be calculated to enable relative comparisons between different C_{18} phases. The selectivity factor $\alpha_{TBN/BaP}$ (defined as k'_{TBN} / k'_{BaP}, where $k'=(t_R-t_0)/t_0$, and t_R, and t_0, are the retention times of the analyte and void volume marker, respectively) has been shown to correlate with PAH selectivity and stationary phase type [1,6-8]. It should be noted that while the separation of isomeric PAH mixtures is often achieved with polymeric C_{18} phases (or other stationary phases with component elution PhPh < TBN < BaP), this type of stationary phase may not be the best for a given separation problem. This column evaluation test mixture enables columns to be grouped into well characterized classes to facilitate method development. A classification scheme has been proposed, based on measurement of $\alpha_{TBN/BaP}$ values for experimental and commercial C_{18} columns [1]. Values for $\alpha_{TBN/BaP} \leq 1$ reflect polymeric C_{18} phases, and values for $\alpha_{TBN/BaP} \geq 1.7$ reflect monomeric C_{18} phases. For values $1 < \alpha_{TBN/BaP} < 1.7$, the synthesis scheme is less certain, and may indicate light polymerization with di- or trifunctional reagents, or densely loaded monomeric phases. A listing of commercial C_{18} columns, grouped using this classification scheme, is provided in the Appendix, Table 2.

Relationship Between Retention Order and PAH Selectivity: Two examples of changes in stationary phase selectivity are illustrated in Figures 4 and 5. The separation of PAH isomers of molecular weight 278 (see also Figures 7 and 8) is shown in Figure 4. Selectivity factors for four commercial and experimental columns range from 0.38 to 1.80. The best separation of these isomers was achieved with the most heavily loaded polymeric C_{18} phase, with $\alpha_{TBN/BaP} = 0.38$. The lowest selectivity for these isomers was found with a monomeric phase ($\alpha_{TBN/BaP} = 1.80$). Using the same set of columns, SRM 1647a, Priority Pollutant Polycyclic Aromatic Hydrocarbons in Acetonitrile was separated using gradient elution conditions (see Figure 5). The current renewal of this material contains the same components at similar mass fraction levels, and is designated SRM 1647d. Separation of this mixture (required by EPA Method 610) should be possible with C_{18} columns having $\alpha_{TBN/BaP}$ values in the range ~0.5 < $\alpha_{TBN/BaP}$ < ~0.9. Separation can also be achieved for $\alpha_{TBN/BaP}$ < ~0.4. Complete separation of all 16 components was possible with the two polymeric C_{18} columns ($\alpha_{TBN/BaP} = 0.65$ and 0.38), but with a reversal in the elution of dibenz-[*a,h*]anthracene and benzo[*ghi*]perylene on the heavily loaded polymeric C_{18} phase. Separation of the components occurred over the widest time interval, with the heavily loaded polymeric C_{18} phase ($\alpha_{TBN/BaP} = 0.38$). Separation of all components is generally not possible for $\alpha_{TBN/BaP}$ > ~0.9; however, by modifying column selectivity by changing chromatographic conditions (e.g., reducing column temperature, see page 4), baseline separation of all 16 components can be achieved.

DISCUSSION

The selectivity factor $\alpha_{TBN/BaP}$ has been shown to vary with stationary phase type [6], substrate properties [9], alkyl chain length of bonded ligand [10], stationary phase density [7], mobile phase composition [5] and column temperature [4]. In general, column selectivity for complex PAH mixtures (such as PAH isomers) increases with decreasing $\alpha_{TBN/BaP}$. Furthermore, separations often can be reproduced under different conditions (i.e., different combinations of stationary phase type, alkyl chain length, column temperature, and mobile phase composition) as long as $\alpha_{TBN/BaP}$ is held constant. Thus by adjusting these chromatographic variables, differences in column selectivity among columns from different manufacturers may be compensated.

In practice, stationary phase type, alkyl chain length, and pore size are most easily varied by changing columns. As column manufacturers begin to provide $\alpha_{TBN/BaP}$ data on their C_{18} columns, column selection and method development will be facilitated. A few trends, however, can be summarized.

Stationary Phase Type: The single most important parameter affecting column selectivity is bonded phase chemistry [6]. As described above, stationary phases prepared using polymeric surface modification chemistry will usually have $\alpha_{TBN/BaP}$ values < 1, and will exhibit increased selectivity for PAH isomers compared to monomeric C_{18} phases ($\alpha_{TBN/BaP}$ > 1.7, Figure 3). Stationary phases prepared using limited polymeric syntheses, or prepared using highly reactive monomeric syntheses may result in intermediate properties, (i.e., $1 < \alpha_{TBN/BaP} < 1.7$).

Pore Size: Stationary phase selectivity is also dependent on pore size of the silica substrate. For polymeric C_{18} phases, the ability to separate PAH isomers (shape selectivity) increases with increasing pore size [9]. For monomeric C_{18} phases, little dependence is observed between column selectivity and pore size. For polymeric C_{18} phases, however, selectivity shifts with changes in pore diameter, becoming more shape selective (more polymeric-like) with increasing pore size. Benz[*a*]anthracene and chrysene coelute on 6 nm pore diameter polymeric C_{18} columns, but are fully resolved on 30 nm pore diameter polymeric C_{18} columns. Similarly, benzo[*ghi*]perylene and indeno[1,2,3-*cd*]pyrene are unresolved on the narrow pore polymeric C_{18} columns, but are resolved on the wide pore polymeric C_{18} columns. For both monomeric and polymeric C_{18} phases, these trends are reflected in the retention behavior of the column selectivity test mixture.

Stationary Phase Length: Changes in column selectivity are also observed for stationary phases with different alkyl chain lengths [10]. In general, shape selectivity increases with increasing alkyl chain length. The elution order for the selectivity test mixture is as expected for the monomeric and polymeric C_{18} phases. Benzo[*a*]pyrene elutes earlier than normal on stationary phases shorter than C_{18} and longer than normal on phases longer than C_{18}. This trend is summarized in Figure 6 as a plot of selectivity ($\alpha_{TBN/BaP}$) vs. stationary phase length. It is interesting to note that stationary phase selectivity of monomeric and polymeric phases merge at short and long alkyl chain lengths. Monomeric and polymeric C_8 phases have similar selectivity toward PAH, as do monomeric and polymeric C_{30} phases. Again, the selectivity factor $\alpha_{TBN/BaP}$ is indicative of overall stationary phase selectivity for more complex PAH mixtures [10].

Stationary Phase Coverage: Among polymeric C_{18} phases, selectivity has been shown to vary with the density or coverage of the polymeric layer [7]. A similar but more subtle trend has been demonstrated for monomeric C_{18} phases [11]. Shape selectivity for PAH isomers increases with increasing stationary phase density. This trend is illustrated for a series of PAH isomers (MW 278) in Figures 7 and 8, for which retention increases with increasing solute length-to-breadth [12]. Baseline resolution of the isomers was achieved on a heavily loaded polymeric C_{18} phase, but only partial resolution was possible on lower loaded polymeric C_{18} phases. Very little resolution of the isomers was achieved on monomeric C_{18} columns.

Temperature: Temperature can have a significant effect on stationary phase selectivity [4]. PAH shape recognition increases dramatically with decreasing temperature. Conversely, shape recognition decreases with increasing temperature. These trends are reflected in the retention behavior of the selectivity test mixture as illustrated in Figure 9. The relative retention of BaP increases with decreasing temperature, indicating an increase in shape selectivity. This behavior has been verified for several complex PAH mixtures, including SRM 1647a and the six methylchrysene isomers [4]. Although Figure 9 illustrates temperature effects for a polymeric C_{18} column,

the change in selectivity with temperature is a general phenomenon that is not dependent on stationary phase type or column manufacturer.

The dependence of selectivity on temperature for a typical monomerically bonded and polymerically bonded column is summarized in a plot of $\alpha_{TBN/BaP}$ vs. temperature in Figure 10. Changes in selectivity are relatively uniform for the polymeric C_{18} phase. In contrast, changes in selectivity for the monomeric C_{18} phase are most significant at subambient temperatures; selectivity is nearly constant above 25 °C. Two possibilities are suggested by this plot. It should be possible to duplicate "polymeric-like" selectivity with a monomeric column, by performing the separation at reduced temperature. This possibility has been demonstrated for the separation of SRM 1647a [4]. Furthermore, it should be possible to achieve column selectivity characteristics not possible with available columns, (i.e., by cooling polymeric C_{18} columns). This possibility has been demonstrated for a separation of methylchrysene isomers which were previously not separable by liquid chromatography [4]. Thus, column selectivity may be adjusted as required by altering column temperature. Although the temperature dependence of $\alpha_{TBN/BaP}$ will vary for individual columns, the plots in Figure 10 provide a basis for tentative predictions.

Application to Carotenoid Separations: Many of the selectivity trends described above for PAH isomers are also observed for carotenoids [3,13]. As with PAH isomers, better separations of carotenoid isomers usually result from the use of polymeric C_{18} phases compared with monomeric phases. In addition, stationary phases prepared with long (C_{30}) alkyl chains have enhanced selectivity toward carotenoids [13]. These properties are indicated by $\alpha_{TBN/BaP} < 1$. It should be noted that other stationary phase properties not indicated by SRM 869a may also be important in the assessment of column suitability for carotenoid separations. For example, silanol activity has been shown to influence the separation of xanthophylls (polar carotenoids) [13].

Certain commercial equipment, instruments, or materials are identified in this certificate to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose. Tabulations of commercial LC columns are not intended to be all inclusive.

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Users of this SRM should ensure that the certificate in their possession is current. This can be accomplished by contacting the SRM Group at: telephone (301) 975-6776; fax (301) 926-4751; e-mail srminfo@nist.gov; or via the Internet http://www.nist.gov/srm.

APPENDIX

The following supplementary information is supplied for the convenience of the user of this material. This information does not meet the requirements for certification by the National Institute of Standards and Technology.

Descriptive Information

This information is provided as an aid for peak identification. The values listed are not intended for quantitative comparisons.

Table 1. Gravimetric Concentrations and Relative Responses at 254 nm

Compound	Mass Fraction (µg/g)	Relative Response[a]									
		A		B		C		D		E	
BaP	2.1	1	[1]	1	[1]	1	[1]	1	[1]	1	[1]
PhPh	3.2	1.46	[1.65]	1	[1]	1.91	[1.46]	0.52	[0.34]	3.00	[1.68]
TBN	11.2	2.30	[4.01]	1.08	[1.42]	3.09	[3.66]	1	[1]	6.11	[4.05]

[a]These measurements refer to the separations in Figure 2 (A-E). Values represent peak heights and peak areas (areas are shown in brackets) relative to BaP, and are listed only as an aid for peak identification.

Table 2. Selectivity Coefficient Ranges $\alpha_{TBN/BaP}$ for Various Commercial C$_{18}$ Columns

Column	Manufacturer
$\alpha_{TBN/BaP}$ = 0.3 - 0.9	
Prosphere 300 C18	Alltech
Hypersil Green PAH	Shandon HPLC
Environsep PP	Phenomenex
Supelcosil LC-PAH	Supelco
Baker Wide Pore C18	J.T. Baker
Chromspher PAH	Chrompack
Keystone PAH	Keystone Scientific
Biorad RP 318	Biorad
Supelcosil LC-PAH	Supelco
CP Ecosphere PAH	Chrompack
Exsil PAH	Alltech
Vydac 201TP	The Separations Group
Spherisorb PAH	Phase Separations
$\alpha_{TBN/BaP}$ = 1.0 - 1.7	
Brownlee ODS 5A	Brownlee
Bakerbond C18	J.T. Baker
Zorbax Rx C18	Rockland Technologies
LiChrospher 60 RP-select B	E. Merck
Partisil 5 ODS-2	Whatman
Partisil 5 ODS	Whatman
LiChrospher 100 RP-l8	E. Merck
Spherisorb ODS-l	Phase Separations
Zorbax C18	Rockland Technologies
Spherisorb ODS-2	Phase Separations

Table 2. Con't.

$\alpha_{TBN/BaP}$ = 1.7 - 2.2

Microsorb C18	Rainin
Partisphere C18	Whatman
μBondapak C18	Waters
Spheri 5 RP-18	Brownlee
Cosmosil C18	Nacalai Tesque
Partisil 5 ODS-3	Whatman
Ultracarb 5 ODS (20)	Phenomenex
Nova-Pak C18	Waters
YMC 120 Å "A"	YMC
Capcell C18	Shiseido
Ultrasphere ODS	Beckman
Adsorbosphere HS	Alltech
Ultracarb 5 ODS (30)	Phenomenex
Supelcosil LC - 18	Supelco
ODS Hypersil	Shandon HPLC
Zorbax Eclipse C18	Rockland Technologies
Supelcosil LC-18-DB	Supelco

Comments: Selectivity coefficients were determined using 85 % acetonitrile in water mobile phase at 2 mL/min, at ambient temperature (25 °C ± 2 °C). Columns are listed in order of increasing $\alpha_{TBN/BaP}$ values; however, because these values may vary with different column lots, individual $\alpha_{TBN/BaP}$ values are not listed. A tabulation of these selectivity factors will be provided upon request. The ranges shown above should not be construed to reflect lot-to-lot variability.

BaP

Benzo[a]pyrene, BaP

PhPh

Phenanthro[3,4-c]phenanthrene, PhPh

TBN

1,2:3,4:5,6:7,8-Tetrabenzonaphthalene, TBN
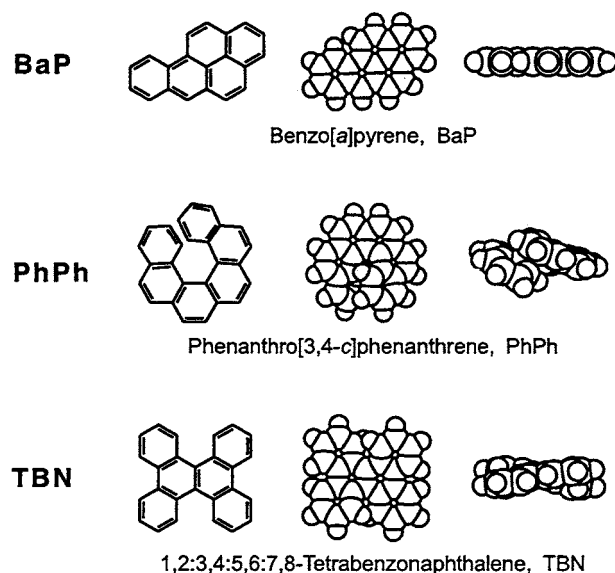
Figure l. Structures and space filling models for compounds in SRM 869a.

A) $\alpha_{TBN/BaP}$ = 2.08 (monomeric)

B) $\alpha_{TBN/BaP}$ = 1.80 (monomeric)

C) $\alpha_{TBN/BaP}$ = 1.27 (intermediate)

D) $\alpha_{TBN/BaP}$ = 1 (polymeric)

E) $\alpha_{TBN/BaP}$ = 0.48 (polymeric)
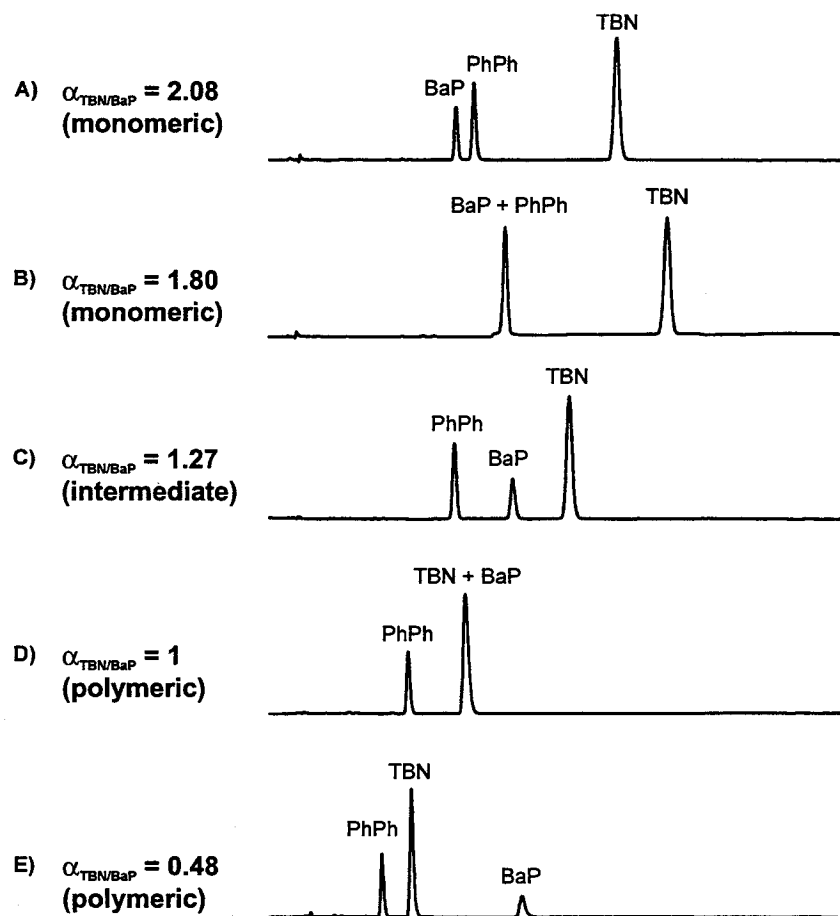
Figure 2. Separation of SRM 869a on dissimilar LC columns. Monomeric and polymeric designations refer to stationary phases prepared with monomeric or polymeric surface modification chemistry; intermediate designation refers to a stationary phase (possibly monomeric or polymeric) with intermediate properties. These chromatograms are provided to aid peak identification.

TBN

PhPh

BaP

TBN

PhPh

BaP

Monomeric C_{18}

$\alpha_{TBN/BaP}$ > 1.7

Low Shape Selectivity

Polymeric C_{18}

$\alpha_{TBN/BaP}$ < 1

High Shape Selectivity
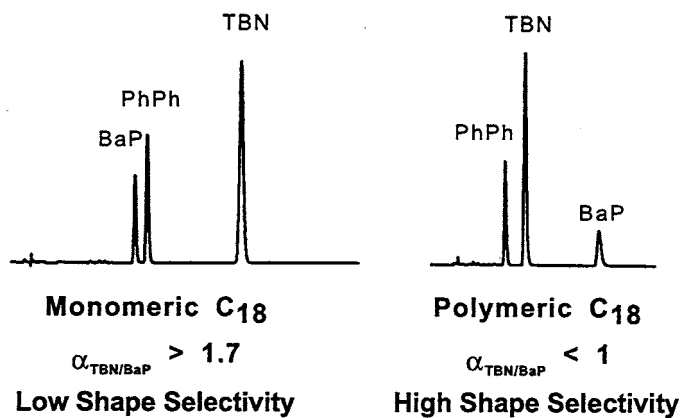
Figure 3. Separation of SRM 869a on C_{18} columns prepared with monomeric and polymeric surface modification chemistry.

A) $\alpha_{TBN/BaP}$ = 1.80
(monomeric)

B) $\alpha_{TBN/BaP}$ = 1.27
(intermediate)

C) $\alpha_{TBN/BaP}$ = 0.65
(polymeric)

D) $\alpha_{TBN/BaP}$ = 0.38
(polymeric)

dibenzo[c,g]phenanthrene
benzo[g]chrysene
dibenzo[b,g]phenanthrene
dibenz[a,c]anthracene
benzo[c]chrysene
dibenz[a,j]anthracene
pentaphene
dibenz[a,h]anthracene
benzo[a]naphthacene
benzo[b]chrysene
picene

Figure 4. S   neric and
polymeric su

A) $\alpha_{TBN/BaP}$ = 1.80
(monomeric)

naphthalene
acenaphthylene
acenaphthene + fluorene
phenanthrene
anthracene
fluoranthene
pyrene
benz[a]anthracene + chrysene
benz[b]fluoranthene
benz[k]fluoranthene
benzo[e]pyrene
dibenz[a,h]anthracene
indeno[1,2,3-cd]pyrene
benzo[ghi]perylene

Figure 5. Separation of SRM 1647a on the same C_{18} columns as in Figure 4. Complete separation of all 16 components was only possible with the C_{18} columns prepared using polymeric surface modification chemistry ($\alpha_{TBN/BaP}$ = 0.65 and 0.38).
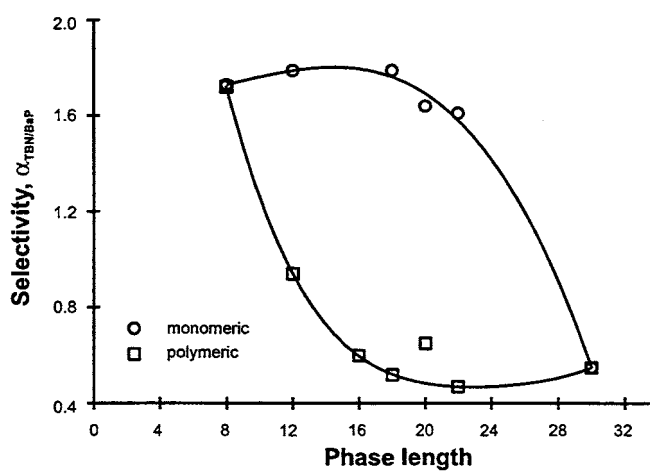
Figure 6. Plot of column selectivity ($\alpha_{TBN/BaP}$) vs. alkyl chain length for stationary phases prepared using monomeric and polymeric surface modification chemistry.

	Structure	Name	L/B
1		Dibenzo[c,g]phenanthrene	1.12
2		Benzo[g]chrysene	1.32
3		Dibenzo[b,g]phenanthrene	1.33
4		Dibenz[a,c]anthracene	1.24
5		Benzo[c]chrysene	1.47
6		Dibenz[a,j]anthracene	1.47
7		Pentaphene	1.73
8		Dibenz[a,h]anthracene	1.79
9		Benzo[a]naphthacene	1.77
10		Benzo[b]chrysene	1.84
11		Picene	1.99

Figure 7. Structures and associated length-to-breadth ratios[10] for PAH isomers of molecular weight 278.
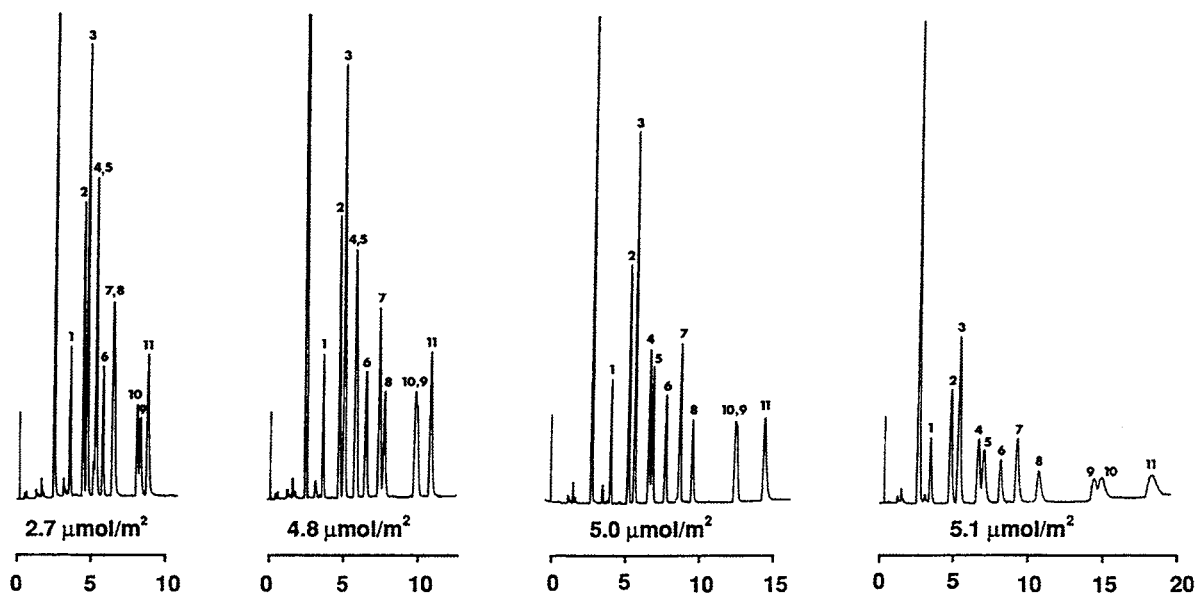

Figure 8. Separation of molecular weight 278 isomers on polymeric C_{18} columns with different stationary phase loadings. Loadings are indicated in terms of surface coverage values, $\mu mol/m^2$. See Figure 7 for compound identification.
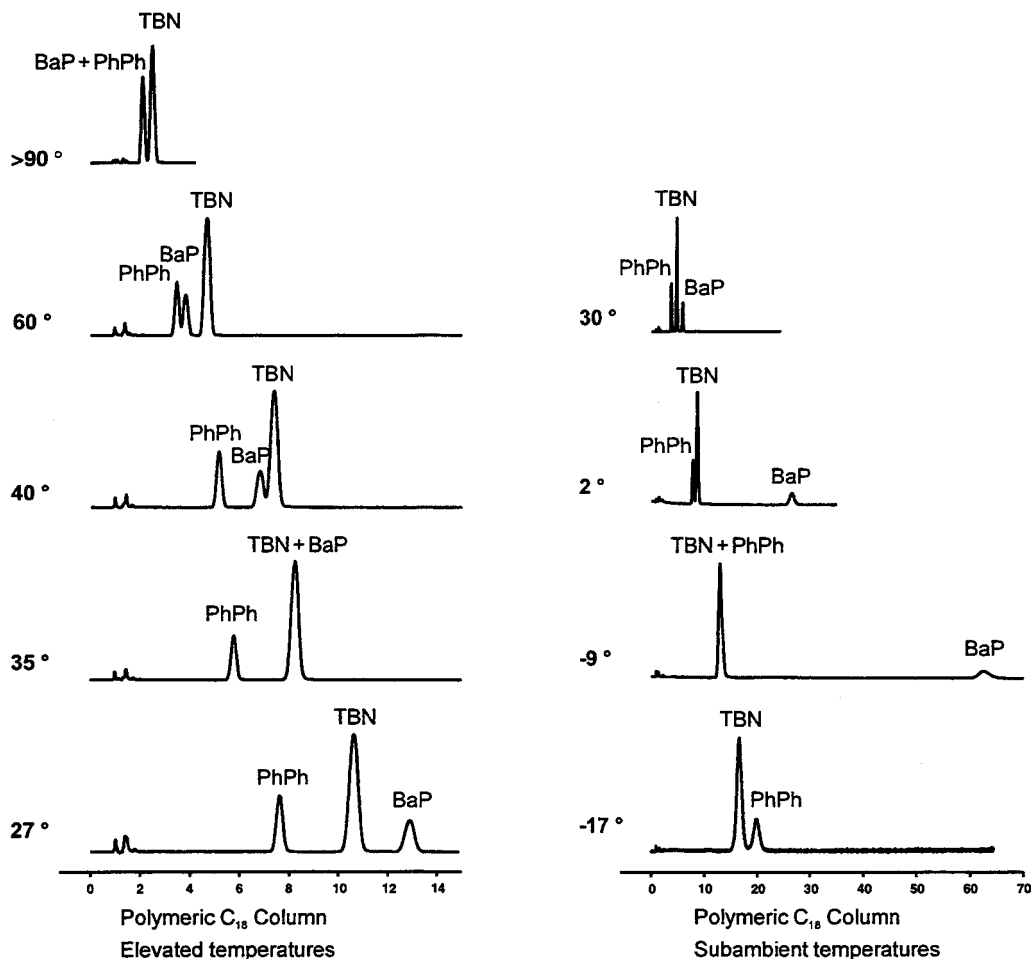
Figure 9. Separation of SRM 869a on a polymeric C_{18} column at different temperatures. Subambient separations were carried out using 85 % acetonitrile/water; separations at elevated temperatures were carried out using 75 % acetonitrile.
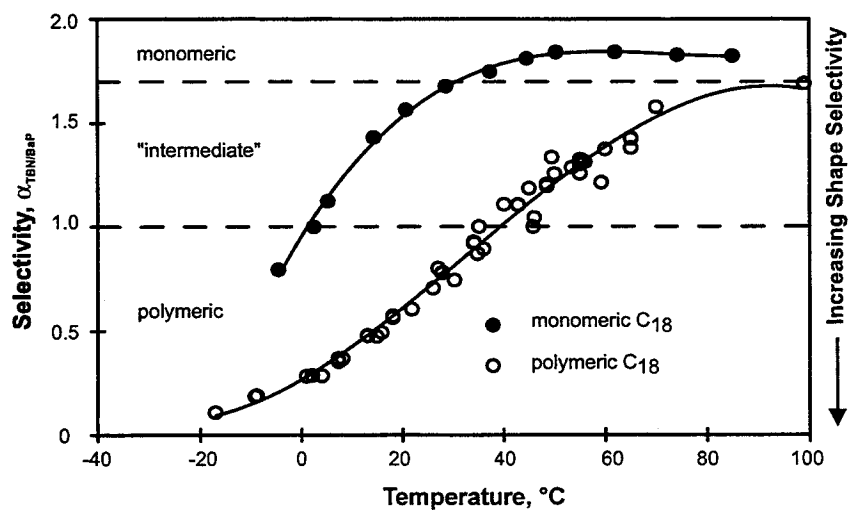

Figure 10. Stationary phase selectivity ($\alpha_{TBN/BaP}$) plotted as a function of temperature for monomeric and polymeric C_{18} columns: Mobile phase, Zorbax C_{18} 85:15 acetonitrile/water; Vydac 2O1TP C_{18} various acetonitrile/water compositions.